EXZACT™ Precision Technology: Scientific and Regulatory Advancements in Plant-Genome Editing with ZFNs

Gary Rudgers and Lakshmi Sastry-Dent
Dow AgroSciences
Indianapolis, Indiana

gwrudgers@gmail.com

Approaches to improving agricultural crops have relied on either mutational or traditional breeding and selection methods or through the random introduction of novel genes into crops by transformation (*e.g.* using *Agrobacterium*). These approaches are inherently time-consuming, inefficient and expensive, especially for developing complex, multi-trait products that are now standard for the industry. To better serve the market, new technologies have been developed that focus on targeted genome editing. One such technology is Dow AgroSciences' EXZACT™ Precision Technology that facilitates precise changes in plant genomes including DNA deletions, edits (point mutations) and targeted gene additions (trans-, cis-, intra-). Using EXZACT™ Precision Technology, developers and breeders can now introduce genetic traits more efficiently and precisely than ever before.

EXZACT™ Precision Technology is based on zinc-finger nucleases (ZFNs) that are composed of a DNA-binding domain (zinc-finger proteins) and a nuclease domain derived from the restriction endonuclease *Fok1.* DNA binding is mediated by ZF modules, where each ZF recognizes a specific three-base-pair DNA sequence. Typically four to six individual ZFs are linked together for specific recognition of a unique DNA sequence (12–18 nucleotides) (Figure 1). The *Fok1* nuclease that generates the specific double-stranded DNA cleavage at the targeted DNA sequence requires dimerization of the two *Fok1* domains for cleavage activity. Therefore, functional ZFNs are dimeric proteins consisting of two unique zinc-finger proteins (Figure 1) that bind to two unique DNA

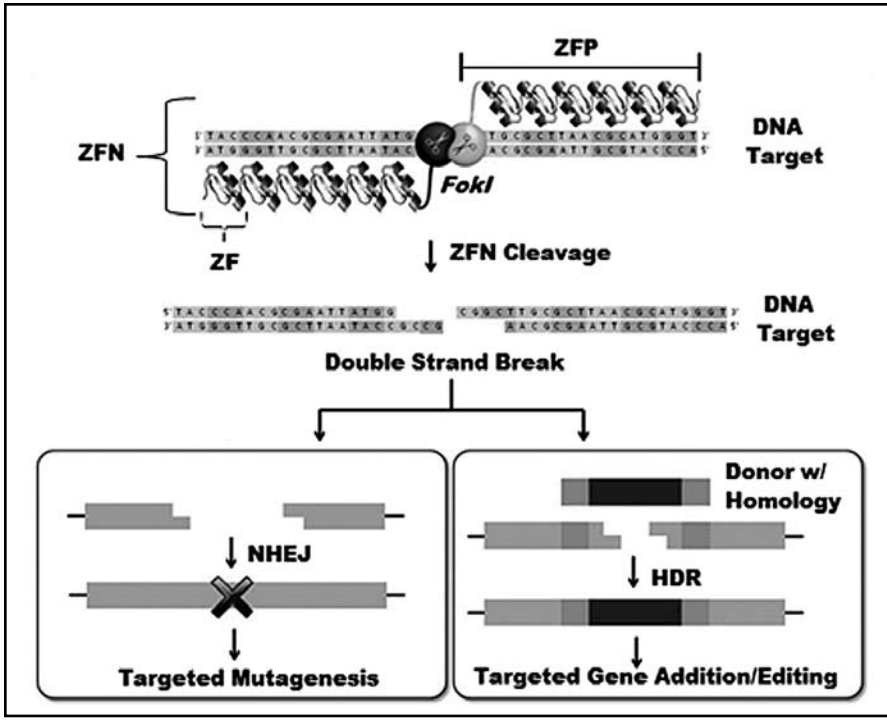

Figure 1. Model of ZFN DNA cleavage and repair in the presence (HDR pathway) or absence (NHEJ pathway) of a DNA-repair template. Depending on the DNA-repair pathway and repair donor-DNA template, genomic deletions, edits or additions can be generated, screened and selected.

sequences (24–36 nucleotides) in the correct orientation, at the same time, to generate a targeted double-strand DNA break.

The EXZACT™ Precision Technology platform exploits the functionality of ZFNs and plant cellular DNA-repair mechanisms to facilitate targeted deletions (*ZFN-1*), editing (*ZFN-2*) and addition (*ZFN-3*) of DNA (or genes) in model and specialty-crop species. When ZFNs are delivered into plant cells, they bind to a specific, predetermined genomic sequence and introduce a DNA double-strand break that activates the innate cellular DNA-repair pathways. Repair by non-homologous end-joining (NHEJ) may result in the creation of small insertions or deletions (INDELS) at the break site, leading to targeted mutagenesis and effective disruption of the genomic sequence (Figure 1). If the initial target sequence is a gene, NHEJ-induced targeted mutagenesis leads to loss-of-function or altered-function alleles. The double-stranded DNA breaks can also be repaired by homology-directed repair (HDR) in the presence of a donor DNA molecule with homology to the break site, resulting in targeted donor addition. With the HDR, presence of a gene of interest (GOI) in the donor leads to targeted gene addition, whereas point mutations in the donor give rise to a genome-editing mutation (Figure 1).

Should one of the text references be Figure 2 instead of 1?

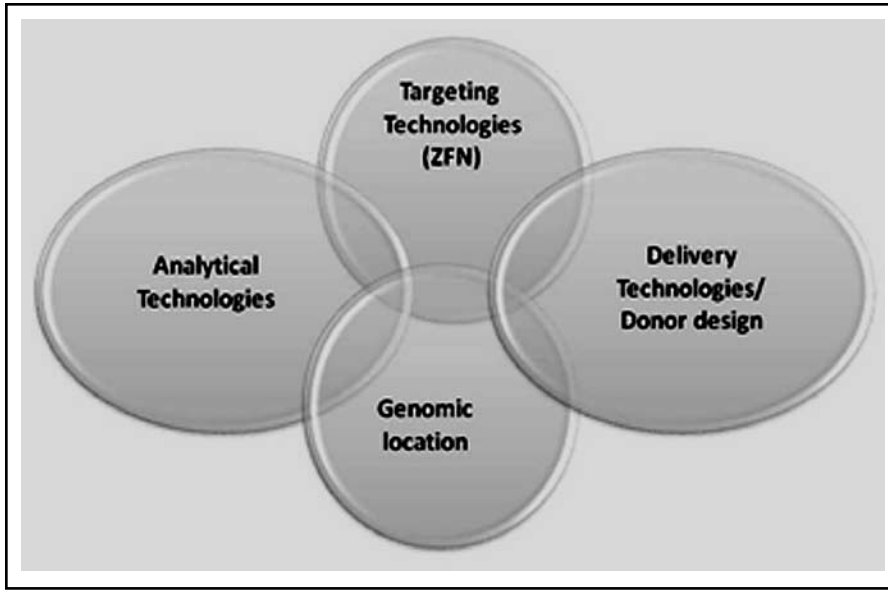

Figure 2. EXZACT™ Precision Technology platform. Donor design, delivery, analytics and genome-location information was coupled with DNA double-strand break generation ability to create an operable platform for trait applications.

EXZACT™ targeted gene addition offers several benefits for trait-product development compared to conventional methods. With current transformation methods, a GOI is randomly inserted in the plant genome and screening for events of interest is time-, resource- and cost-intensive. In contrast, targeted gene addition to a specific, desired locus, such as a safe harbor location (see *Genomic Locations* below) is expected to generate higher-quality events with minimal unintended side-effects and an increased probability of achieving the desired product. In addition, targeting to a specific locus simplifies analytics involved in event characterization and sorting, thus reducing trait-development cycle times.

An additional advantage of gene addition is that GOIs can repeatedly be targeted to the same safe-harbor location to generate new events, reducing costs over time through the reuse of targeting reagents, analytics and potentially regulatory and introgression processes. Gene addition also provides new options for stacking, to generate multi-gene, multi-trait products. Currently, stacking traits is primarily achieved by breeding together multiple traits from single events located at various loci in the plant genome. However, as the number of traits being bred or stacked into a single product increases, efficiency of stacking decreases due to the increased likelihood of the various loci segregating during the breeding process. Using ZFNs, it is possible to target multiple genes to the same location, which decreases the number of loci involved in breeding and facilitates multi-trait product development. It is also feasible to use ZFNs to add genes to existing commercial-grade events to generate new gene stacks to meet customer and market needs.

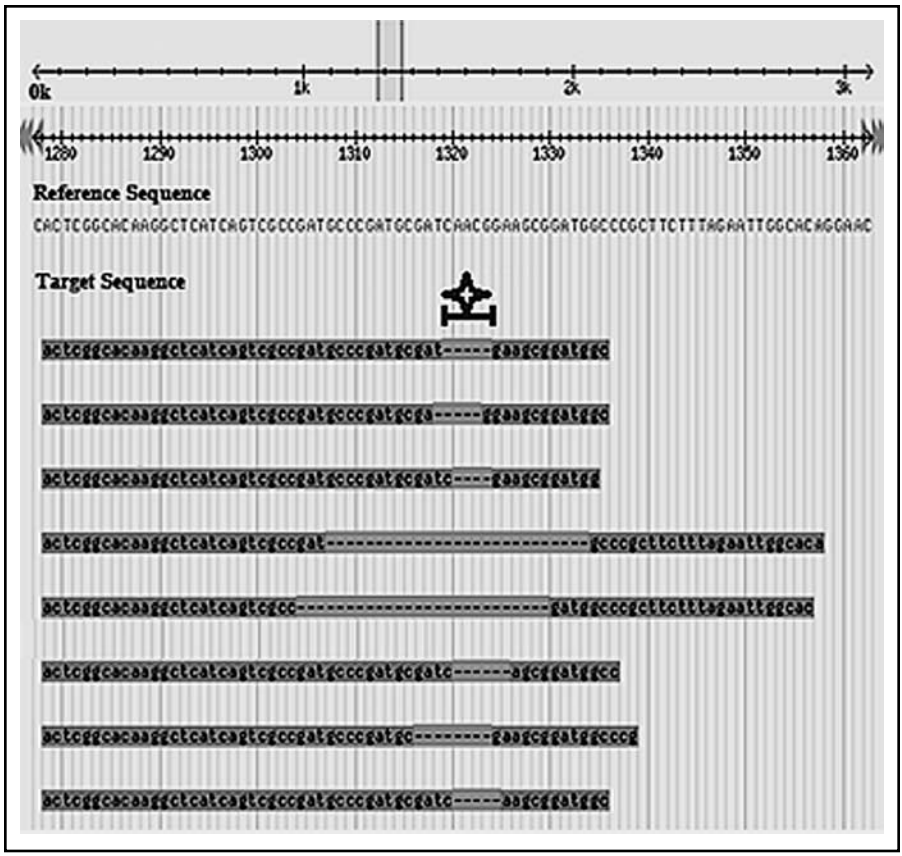

Figure 3. Activity of a ZFN in maize cells. DNA-cleavage activity of ZFNs is measured by the presence of INDELs (-----) using illumine sequencing. The bar with the star represents the cleavage site for the ZFN. The reference target sequence is shown at the top and sequences with INDELs from Illumina® are shown in dark gray.

EXZACT™ Precision Technology Platform

To optimize and enhance EXZACT™ technology for trait-product development, an EXZACT™ Precision Technology platform has been developed (Figure 2). This platform consists of companion technologies including DNA-donor design and delivery, analytics, identification and optimization of gene-targeting genomic locations and advancements in ZFN-targeting technology. The combined suite of technologies making up the EXZACT™ Precision Technology platform has been developed in several crop species including maize, soybean, canola and wheat.

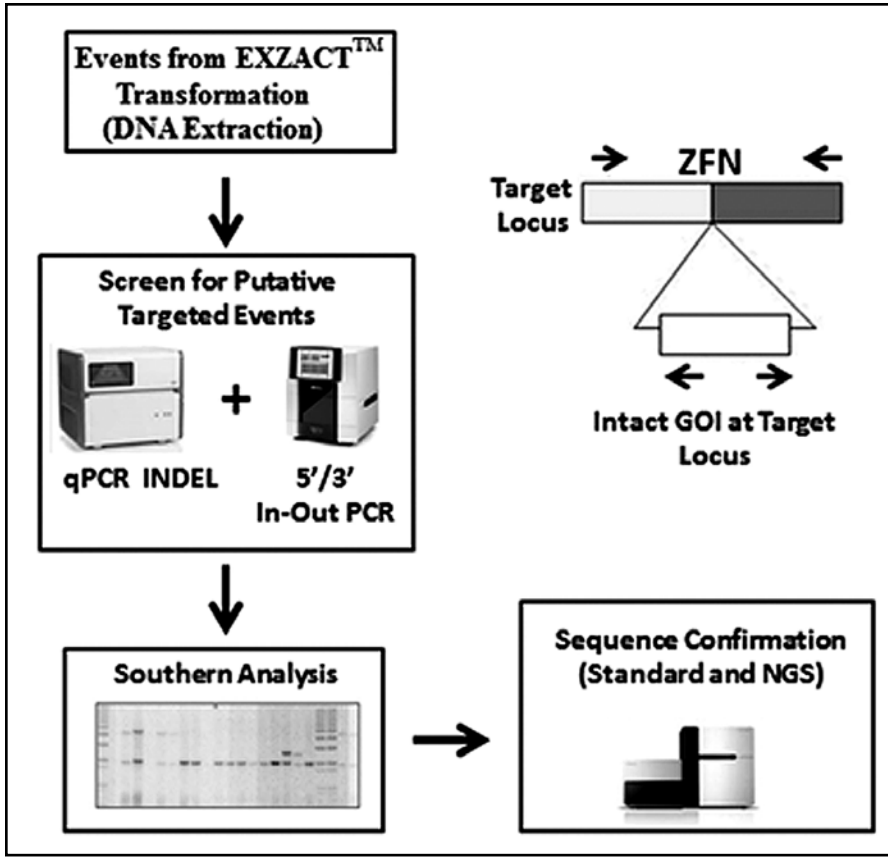

Figure 4. Analytical process for identifying targeted events.

Targeting Technologies: ZFN Design and Testing

ZFN design and testing capability is critical for a robust genome-editing program. Functional ZFNs have been developed for targets in a number of model and crop species for gene addition, deletion and editing applications (Cai *et al.*, 2009; Shukla *et al.*, 2009; Ainley *et al.*, 2013).

With advancements in ZF designs, ZFNs can now be effectively developed for DNA targets less than 100 bp and have been linked to enhanced (obligate heterodimeric) *Fok1* nuclease domains to reduce the potential of off-target cleavage (Doyon *et al.*, 2011). An agreement between Dow AgroSciences and Sigma Aldrich has been developed for plant-ZFN design and supply to meet product-scale needs.

In addition to improved ZFN designs, prior to deploying EXZACT™ ZFNs in plants, 3–16 ZFN designs are typically generated per DNA target and assayed for their ability to generate targeted DNA double-stranded breaks. Lead-candidate designs are identified by transient expression in plant cells followed by deep sequencing of genomic DNA from ZFN-treated and control materials. Delivery of ZFNs into cells leads to DNA double-

strand breaks that are repaired by NHEJ, which leaves an INDEL footprint at the break site. Presence of, and number of, sequences with INDELS is a measure of ZFN activity. A diverse INDEL footprint, as seen by the presence of different types of deletions, further indicates effectiveness of a ZFN. A representative activity profile for a maize target-specific ZFN is shown in Figure 3.

As a result of improved EXZACT™ ZF design, use of obligate heterodimeric *Fok1* nucleases and screening of designed ZFNs for DNA-binding and -cleavage activity, the resulting EXZACT™ ZFNs deployed for gene deletion, addition or editing result in highly efficacious ZFNs with minimal off-target cleavage and superior results.

Analytical Technologies

Analytical technologies are foundational to the success of the EXZACT™ technology. In most plant-cell-targeting studies, targeted gene integration occurs in conjunction with random integration. Therefore, there is a need for sensitive methods for rapid identification of only targeted events. A tiered molecular process has been developed that uses two PCR-based screens for rapid identification of putative targeted events that are then confirmed by higher resolution methods, including Southern analysis and next-generation sequencing (Figure 4). PCR screens are designed to measure both intact ZFN-binding sites at a target locus and also score for the presence of the GOI at the target site by junctional in-out PCR (Ainley *et al.*, 2013). The duplicate screens are done in parallel to reduce false positives and increase the robustness of targeted event identification.

Delivery and Donor-Design Technologies

Ability to effectively co-deliver ZFN and donor constructs is critical for achieving targeted genome addition. In maize, targeted gene addition has been achieved successfully by delivering ZFN and donor constructs using WHISKERS™ and microparticle bombardment (Shukla *et al.*, 2009; Ainley *et al.*, 2013). In addition to DNA delivery, donor constructs have been optimized for efficient and optimal HDR of the targeted double-stranded break through the use of 750- to 1,000-bp homology sequences flanking the GOI.

Genomic Location

In a plant genome, not all locations are suitable for targeted gene insertion. Safe-harbor locations are genomic regions where transgenes can be added with minimal unintended side effects and for consistent, reproducible transgene performance. Such safe-harbor locations can be identified through previously characterized transgenic plants. Event-32 is a Dow AgroSciences' pipeline event that was generated by standard transformation of maize with a corn-rootworm-resistant/herbicide-tolerant gene stack. The event performed well in the field as demonstrated by stable trait expression and efficacy, and exhibited neutral agronomics and good breeding characteristics. Because of these features, the transgene-insertion site of the event was classified as a potential safe-harbor location. This safe-harbor location has since been retargeted in a Hi-II maize background using a custom-designed ZFN (ZFN6) and a GOI by HDR (Figure 5). As a result of improved ZFN designs, screening and analytic methods, targeting frequencies of 2% have repeatedly been achieved at this safe-harbor location.

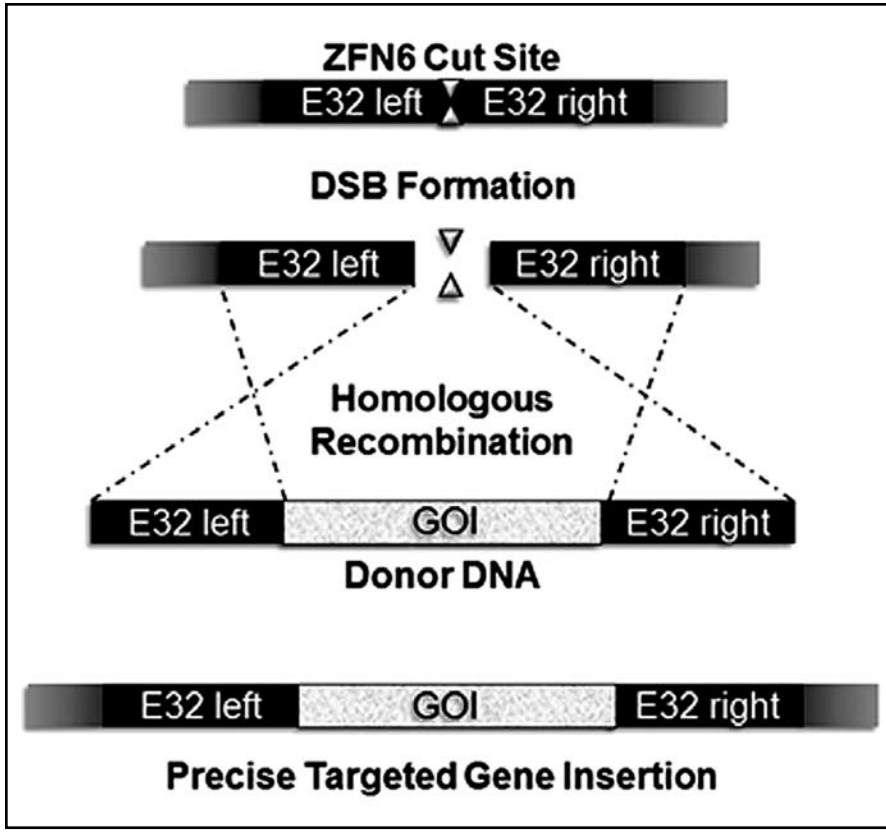

Figure 5. Targeted gene addition at the Event-32 locus.
A GOI was inserted at the E32 locus using EXZAT™ technology.
E32 left/right: sequences homologous to the E32 locus.
ZFN6: E32 locus-specific ZFN.

Gene Stacking

For stacking multiple genes at a single location using EXZACT™ technology, a suite of engineered ZFNs (eZFNs) and EXZACT™ landing pads (ELPs) have been developed (Ainley *et al.*, 2013) (Figure 6). ELPs are unique DNA sequences that carry an eZFN DNA-binding sequence flanked with DNA sequences homologous to an incoming donor DNA sequence. For stacking, an ELP with a first GOI is pre-integrated into the plant genome (*e.g.* E32 safe harbor); subsequent delivery of a donor carrying a second GOI and an appropriate eZFN leads to a DNA double-strand break at the ELP and insertion of the donor via HDR, resulting in stacking of the two genes of interest (Figure 6). Presence of a second ELP in the incoming donor allows addition of a third GOI, and so on. This strategy has been used to stack two herbicide-tolerant genes, PAT and aryloxyalkanoate dioxygenase 1 (AAD-1) in maize. The stacked genes were functional

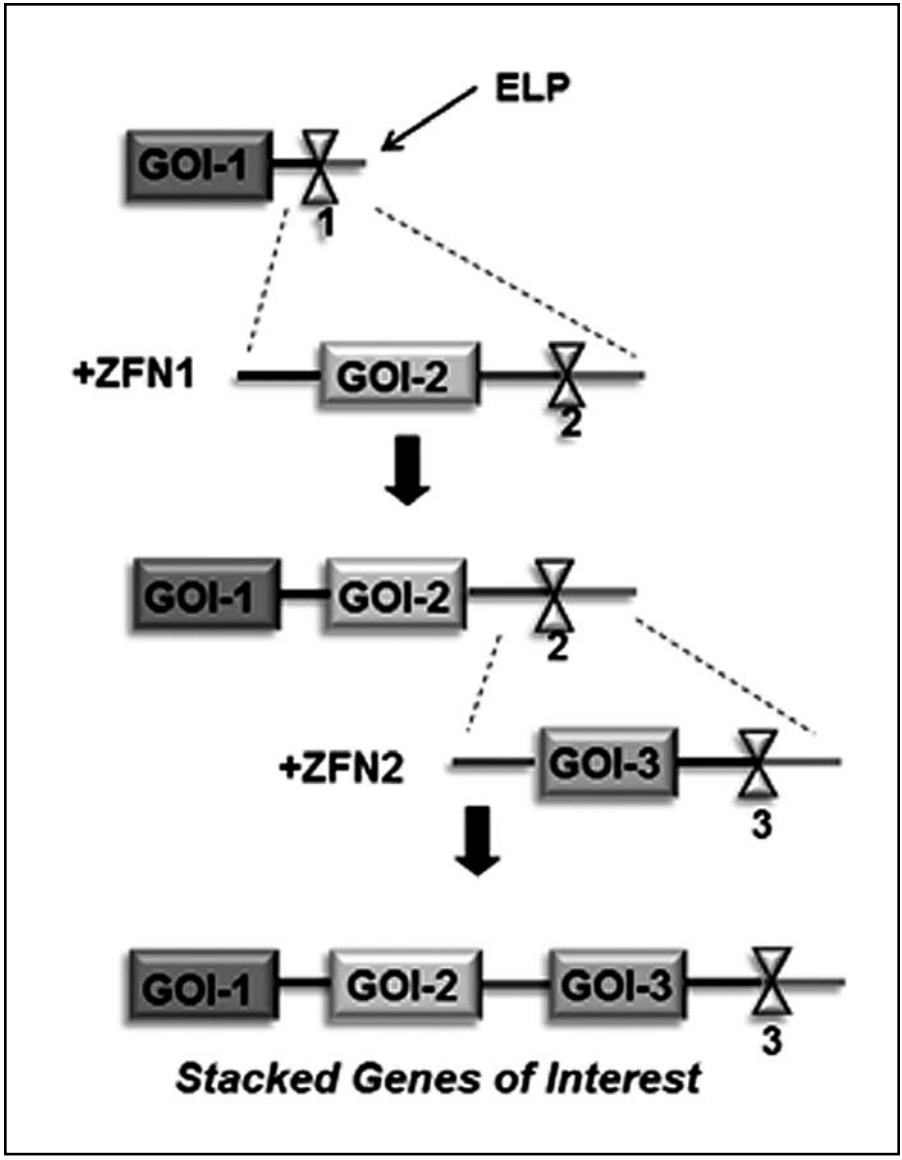

Figure 6. Transgene stacking with EXZACT™ landing pads.

at expression and phenotypic levels and co-segregated as expected (Ainley *et al.*, 2013). The stacking strategy allows insertion of multiple genes at a single location to facilitate multi-trait product development.

The EXZACT™ Precision Technology platform is a new way of thinking about agricultural biotechnology, and represents a departure from conventional breeding

methods. Targeted gene deletion, editing and addition have been demonstrated in tobacco, maize, canola, tomato and wheat. In maize, targeting to an endogenous locus (IPK1) as well as stacking of herbicide-tolerant traits at an engineered locus have been achieved (Shukla *et al.*, 2009; Ainley *et al.*, 2013).

External Collaborations

EXZACT™ Precision Technology is available and accessible both to the public and private sectors through a Dow AgroSciences' licensing agreement. Like the private sector, public-sector breeders and scientists have significant opportunities to employ EXZACT™ Precision Technology in their breeding programs, especially in minor crops. Dow AgroSciences has entered into several licensing agreements with partners around the world to develop targeted gene improvements ranging from deletions, edits and gene insertions in row, community and specialty crops such as maize, canola, cassava, wheat, tobacco, tomato and forestry trees.

As an example, the Department of Environment and Primary Industries (DEPI) of the State of Victory, Australia, through its commercial arm, Agriculture Victoria Services Pty Ltd. (AVS), strengthened a collaborative agreement to improve the performances of Australian canola varieties. The project uses the EXZACT™ Precision Genome Editing Technology platform to continue developing new varieties of canola with enhanced performance designed to benefit farmers in Australia and globally. In addition, AVS will also use the EXZACT™ Precision Genome Editing Technology platform to enhance the genetics of crops important to Australian primary producers.

Through valuable collaborative efforts, a variety of improved crop varieties is being developed with value-adding traits ranging from more-nutritious and insect-resistant cassava and higher-yielding tomatoes, to oil crops with healthier, improved oil profiles and crops with improved herbicide tolerance.

Advances in custom ZFN designs, high resolution analytics, novel donor designs, delivery technologies and genomics will continue to expand the utility of the EXZACT™ Precision Technology for trait discovery and product development. Benefits of targeting genes, genomic deletions and edits at desired locations in plant genomes will continue to be realized, resulting in reduced cycle times and costs for developers while resulting in improved, high-value crops for the farmer and consumer.

Regulatory Considerations

ZFN applications are simply innovative improvements and refinements of traditional breeding methods used to optimize plant health, nutritional quality and yield. Changes and variation in plant genomes (*e.g.* mutations, chromosome rearrangements) are ubiquitous and essential drivers for plants to adapt to their environment and have resulted in a wide variety of crops with a long history of safe use. In fact, over the past century, mutagenesis applications (*e.g.* chemical and radiation mutagenesis) have resulted in over 3,200 beneficial crop varieties that have been planted and safely consumed worldwide in over 175 species (IAEA, 2010). In addition, once developed, traditional breeding processes are deployed to efficiently breed desired mutations into elite plant varieties while minimizing the donors' genetic background.

Due to the controlled, precise manner of the EXZACT™ Precision Technology platform, only the desired genomic changes (gene introduction, deletion or edit) are introduced into the plant genome and the resulting trait can be bred into commercial crops more efficiently and rapidly compared to traditional breeding and mutational applications.

In addition to the considerable research and development advantages, EXZACT™ Precision Technology offers potentially significant regulatory advantages for products resulting from both targeted gene addition (ZFN-3) and targeted mutations (ZFN-1,-2). Globally, regulatory oversight of biotech products is a time-consuming and expensive endeavor estimated at $35 million with an average of six years for regulatory approval / deregulation (Phillips McDougall, 2011). As a result, most biotech crops developed to date have been limited to a handful of traits and high production-acre commodity crops where a return on the investment can be realized (James, 2013).

Through targeted precise addition (ZFN-3), unintended effects (yield drag, gene silencing) often associated with random gene integration, can be avoided. By precisely knowing the location of the introduced trait in the plant's genome, EXZACT™ will help researchers and regulators better understand the end product and help develop a more efficient, less costly and less time-consuming regulatory process (FSANZ, 2013).

Like traditional mutagenesis techniques, EXZACT™ Precision Technology delete (ZFN-1) and edit (ZFN-2), can induce changes in the DNA analogous to chemical mutagens, but with the benefit of targeting the mutation to a predetermined location in the plant genome. Traditional mutagenesis techniques are used to induce point mutations or small insertions / deletions and are intrinsically very non-specific, in which thousands of nucleotides are mutated instead of a desired single nucleotide. Additionally, traditional mutagenesis techniques can often result in genetic alterations at multiple genomic locations. As a result, years of breeding are required to eliminate undesired mutations and to cross the desired mutation into an elite commercial crop variety.

Through EXZACT™ Precision Technology delete (ZFN-1) and edit (ZFN-2) applications, breeders can now take advantage of a plant's own natural genetic variability, allowing precise mutations to be made at predefined locations in the genome, resulting in beneficial, desired mutational traits that can be bred into commercial crops more rapidly compared to traditional mutational applications. As with traditional mutational products, EXZACT™ Precision Technology mutational applications do not involve recombinant DNA and result in products that are similar, and in many cases indistinguishable, from conventionally bred or traditional mutagenesis products.

Based on these facts, it has been concluded by USDA-APHIS that EXZACT™ Precision Technology delete products fall outside their scope of regulation (USDA APHIS, 2014). In addition, an Australian scientific panel convened by Food Standards Australia New Zealand concluded that ZFN-1 and ZFN-2 do not present a significantly greater food-safety concern than other forms of mutagenesis. Such changes are small and definable with predictable outcomes and the panel concluded that food derived from such applications would be similar to food produced using traditional mutagenic techniques, and should, therefore, not be regarded as GM (FSANZ, 2013).

Efforts are underway to help facilitate and assist global regulatory decision making around products developed through various gene-editing applications. Gene-editing

techniques used to develop new plant varieties do not pose a specific safety hazard; rather, the characteristics of the plant determine its safety. Thus, the need to regulate plants developed through gene-editing techniques should be driven by the characteristics of the product (*i.e.* whether it is materially different from existing products present in food, feed or the environment) rather than by the method or process used to make that product. In addition, governments should avoid regulating products developed through gene-editing applications that are similar to, or indistinguishable from, products resulting from traditional breeding methods, since they do not differ in their safety (CropLife International, 2014).

Acknowledgments

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GARY RUDGERS is the Global Regulatory Leader for New Business at Dow AgroSciences in Indianapolis. Over the past six years with the company, he has worked to develop, assess and coordinate global regulatory strategies for emerging and leading biotech crops developed through novel plant technologies.

In addition to his role at Dow AgroSciences, **DR. RUDGERS** chairs the CropLife International New Breeding Techniques (CLI-NBT) working group. Since 2013, this industry organization team has developed and distributed documentation on precision-breeding applications to governments and regulatory bodies worldwide. As chair, he has represented the CLI-NBT working group at various international organizations, on government agencies and at international conferences.

Prior to joining Dow AgroSciences in 2008, Rudgers was one of the leading scientists to help launch the biotechnology company, Chromatin, Inc., in Chicago. From 2002 to 2008 he led the Chromatin molecular biology research team in the development of the first autonomous plant mini-chromosomes. Rudgers held a post-doctoral fellowship at the University of Chicago from 2001 to 2002 and received his PhD in molecular microbiology and immunology from Baylor College of Medicine in Houston in 2001.